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16. ABSTRACT

The present study reports the existence of Purkinje cell-specific phosphoprotein, M_r260,000 (PCPP-260), a neuronal membrane phosphoprotein, in cerebellar Purkinje cells. PCPP-260, which on sodium dodecyl sulfate-polyacrylamide gel electrophoresis has an apparent molecular mass of 260,000 Da, has been found to be phosphorylated in particulate preparations by endogenous or added exogenous cyclic AMP-dependent protein kinase, but not by cyclic GMP-dependent, calcium/calmodulin-dependent or calcium/phospholipid-dependent protein kinases. The protein has been found in high concentrations in all mammalian cerebella so far analyzed, including human cerebellum. One- and two-dimensional electrophoretic and peptide mapping analyses of proteins in other brain regions show that a cosely related 265,000 Da phosphoprotein also exists, albeit in low concentrations, outside the cerebellum. Analysis of cerebella from mutant mice, deficient in either Purkinje cells or in granule cells, indicates that PCPP-260 within the cerebellum is restricted to Purkinje cells. Furthermore, subcellular fractionation of rat cerebella indicates that the protein is an integral membrane protein. The cAMP-regulated phosphorylation of PCPP-260 is presumably involved in membrane functions important to Purkinje cells.

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PCPP-260, A Purkinje Cell-Specific Cyclic AMP-Regulated Membrane Phosphoprotein of *M*, 260,000

S. Ivar Walaas, Angus C. Nairn, and Paul Greengard

Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, New York, New York 10021

The present study reports the existence of Purkinje cell-specific phosphoprotein, *M*, 260,000 (PCPP-260), a neuronal membrane phosphoprotein, in cerebellar Purkinje cells. PCPP-260, which on sodium dodecyl sulfate-polyacrylamide gel electrophoresis has an apparent molecular mass of 260,000 Da, has been found to be phosphorylated in particulate preparations by endogenous or added exogenous cyclic AMP-dependent protein kinase, but not by cyclic GMP-dependent, calcium/calmodulin-dependent or calcium phospholipid-dependent protein kinases. The protein has been found in high concentrations in all mammalian cerebella so far analyzed, including human cerebellum. One- and two-dimensional electrophoretic and peptide mapping analyses of proteins in other brain regions show that a closely related 265,000 Da phosphoprotein also exists, albeit in low concentrations, outside the cerebellum. Analysis of cerebella from mutant mice, deficient in either Purkinje cells or in granule cells, indicates that PCPP-260 within the cerebellum is restricted to Purkinje cells. Furthermore, subcellular fractionation of rat cerebella indicates that the protein is an integral membrane protein. The cAMP-regulated phosphorylation of PCPP-260 is presumably involved in membrane functions important to Purkinje cells.

The second messengers cyclic AMP (cAMP) and cyclic GMP (cGMP) achieve most or all of their effects through activation of specific protein kinases (Greengard, 1978, 1981). Both second messengers appear to be important in the function of neurons in the mammalian cerebellum (e.g. Bloom, 1975; Ferrendelli, 1978; Nairn and Greengard, 1983). However, only a limited number of cerebellar phosphoprotein substrates for cAMP-dependent or cGMP-dependent protein kinase have been characterized (Dolphin and Greengard, 1981; Dolphin et al., 1983; Schlichter et al., 1978, 1980). We recently reported the occurrence of a brain phosphoprotein of apparent molecular mass 260 kDa in rat cerebellum (Walaas et al., 1983a). In the present work we have characterized both the protein kinase responsible for its phosphorylation, demonstrated that the protein is present in primate, rodent, and bovine cerebellum, and studied the cellular and subcellular localization of this protein in the rodent cerebellum. The protein appears to be specifically concentrated in the Purkinje cells, and has therefore been designated PCPP-260 (Purkinje cell-specific phosphoprotein, *M*, 260,000).

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Correspondence should be addressed to S. Ivar Walaas, M.D., Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, 1230 York Avenue, New York, NY 10021.

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Materials and Methods

Materials

γ-^{32}P-ATP was synthesized by the method of Glynn and Chappell (1964). *Staphylococcus aureus* V8 protease was from Miles Biochemicals; trypsin from Worthington; Trasylol from Mobay Chemical Corp.; pepstatin A, 8-bromo-cAMP, and 8-bromo-cGMP from Sigma. Phosphatidylserine (Sigma) was suspended in 20 mM Tris-HCl (pH 7.5), as described by Walaas et al. (1983a). Calmodulin was purified from bovine brain by the method of Grand et al. (1979). The catalytic subunit of cAMP-dependent protein kinase was purified from bovine heart as described (Kaczmarek et al., 1980). The protein inhibitor of cAMP-dependent protein kinase was purified from rabbit skeletal muscle, essentially as described by McPherson et al. (1979). cGMP-dependent protein kinase purified from bovine lung (Walter et al., 1980) was a gift from Dr. Robert Lewis of this laboratory.

Sample preparation

Male Sprague-Dawley rats (150–200 gm body weight) were stunned and decapitated. The brains plus various peripheral organs were rapidly placed in ice-cold 0.32 M sucrose (pH 7.4). The cerebellum and, in some experiments, the cerebral cortex, the neostriatum (caudatoputamen), and several other brain regions were carefully dissected as previously described (Walaas et al., 1983a).

Mice were obtained from Jackson Laboratories (Bar Harbor, ME). The mutants used were Weaver (strain B6CBA $^{+/+}$ A), Nervous (strain C3HeB FeJ), and Purkinje cell degeneration (PCD; strain C57BL 6J). The homozygous mutants were identified by their abnormal gait and smaller size as compared with their littermates. The animals used as controls in the present study were littermates consisting of two-thirds heterozygotes and one-third homozygous wild type. These could not be distinguished by any behavioral means and are collectively termed +/?. Both males and females were used. The mice were killed at 3.5–4 months of age by cervical dislocation. Their brains were rapidly placed in ice-cold 0.32 M sucrose (pH 7.4), and the cerebellum, neostriatum, and cerebral cortex were dissected. The rat and mouse samples were rapidly frozen and either analyzed immediately or stored at −70°C.

Samples from human and monkey cerebellum were obtained from Dr. T. Bartfai (University of Stockholm, Sweden). The human sample was taken from the cerebellar hemisphere of an 84-year-old, neurologically normal female whose brain had been frozen within 3 hr postmortem and maintained at −70°C until analysis. Monkey cerebella (*Macaca mulatta*) were derived from 5–10-year-old healthy animals. The brains were removed within 2 min postmortem, rapidly frozen, and kept at −70°C until analysis. Bovine cerebella, obtained from a local slaughterhouse, were kept on ice during transport to the laboratory and then frozen at −70°C until analysis.

Weighed tissue samples were thawed and homogenized, with 20 strokes in a glass–Teflon homogenizer rotating at 2100 rpm, in 10 vol ice-cold standard buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol, 1 mM EDTA, and 50 units/ml of the protease inhibitor, Trasylol, and kept on ice while protein concentration was determined with a dye-binding assay (Bradford, 1976). The homogenates were in some cases further separated into total particulate and soluble preparations by centrifugation at 150,000 × *g* for 30 min at 4°C, as previously described (Walaas et al., 1983a, b).

Subcellular fractionation

Subcellular fractions from rat cerebellum were prepared by differential centrifugation at 4°C (Whittaker and Barker, 1972). Rat cerebella were homogenized, with 10 strokes in a glass–Teflon homogenizer rotating at 900 rpm, in 10 vol of ice-cold 0.32 M sucrose in standard buffer. The homogenate was centrifuged at 900 × *g* for 10 min, and the pellet was washed once in the original volume of sucrose/standard buffer to yield a nuclear fraction (P_1). The pooled supernatants were centrifuged at 12,000 × *g* for 20 min to yield a crude synaptosomal fraction (P_2) and a supernatant, which was then centrifuged at 150,000 × *g* for 30 min to yield a crude microsomal fraction (P_3) and a final cytosol fraction (S). All particulate fractions were resuspended by means of glass–Teflon homogenizers in the original volume of standard buffer. Triton X-100 (final concentration, 0.2% vol/vol) was then added to all subcellular fractions to insure solubilization of tissue components. The fractions were kept on ice until protein content and phosphoproteins were analyzed.

Extraction of cerebellar proteins

To study the ability of salt and of detergent to extract cerebellar proteins, microsomal fractions (P_3) or, in some experiments, total particulate fractions were resuspended in ice-cold standard buffer containing either KCl or NaCl (0.15 or 0.5 M), or Triton X-100 (0.5%, vol/vol). The samples were mixed vigorously and kept on ice for 60 min, whereupon 0.5 ml aliquots were centrifuged at 150,000 × *g* for 30 min. The supernatants, which contained the extracted proteins, were collected, and the pellets were resuspended in standard buffer with glass–Teflon homogenizers. The extracts were dialyzed overnight against standard buffer before analysis.

Phosphorylation of proteins in tissue samples

Phosphorylation was carried out as described previously (Walaas et al., 1983a). Aliquots containing 50–150 μg protein were incubated in a standard medium (final volume, 0.1 ml) containing (final concentration) 25 mM Tris-HCl (pH 7.4), 6 mM $MgSO_4$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 2 μM γ-^{32}P-ATP (10–30 Ci/mmol) and other additions as indicated. The samples were preincubated for 60 sec at 30°C. The reaction was initiated by addition of γ-^{32}P-ATP and terminated after 60 sec by addition of a SDS-containing "stop solution" (Ueda and Greengard, 1977); this was followed by boiling for 2 min. The phosphoproteins were then either separated by one-dimensional SDS-PAGE (Laemmli and Favre, 1973) or by the detergent-urea, two-dimensional gel electrophoretic system developed for cell surface proteins by Imada and Sueoka (1980). The gels were fixed, stained, destained, and dried, and phosphoproteins were visualized by autoradiography (Ueda and Greengard, 1977). The apparent molecular weights of protein bands were determined by comparison with standard proteins, as described by Walaas et al. (1983a). For quantitation, specific phosphoprotein bands were cut out of the gels by using the autoradiograms as guides, and ^{32}P content was measured by liquid scintillation spectrometry. In some experiments, the gel pieces were further processed for peptide mapping, using either *S. aureus* V8 protease or trypsin as described by Cleveland et al. (1977) and Huttner and Greengard (1979).

Phosphorylation of proteins in acid extracts

The experimental procedures described above were found satisfactory for the qualitative demonstration of the presence or absence of PCPP-260. However, such procedures were not well suited for precise quantitation of protein phosphorylation, as has been discussed in detail elsewhere (Walaas et al., 1983a). Acid/detergent extraction, which inactivates endogenous protein kinases and phosphatases (Forn and Greengard, 1978) and is therefore suitable for precise quantitative determination of acid-soluble phosphoproteins, was used for such quantitation. In preliminary experiments with membranes that had been phosphorylated with γ-^{32}P-ATP and the catalytic subunit of cAMP-dependent protein kinase, this extraction technique was found to accomplish reproducible solubilization of 50–60% of the ^{32}P-labeled PCPP-260 when Triton X-100 was included during the extraction procedure (not shown).

In brief, tissue was homogenized in standard buffer and total homogenates were incubated at 30°C for 20 min. This treatment is known to dephosphorylate endogenous phosphoproteins (Forn and Greengard, 1978; Rauch and Roskoski, 1984; Walaas and Greengard, 1984). Zinc acetate, 5 mM, was added, and precipitated proteins were collected by centrifugation and extracted with 10 mM citrate-phosphate buffer (pH 2.8) containing 0.2% Triton X-100 and 2 μg/ml Pepstatin A as previously described (Walaas and Greengard, 1984). Protein content was determined in the final extracts (Bradford, 1976), which were then adjusted to equal protein concentration. The phosphoproteins present in equal aliquots of the neutralized acid extracts were quantitated by "back-phosphorylation" in a medium (final volume, 0.1 ml) containing (final concentration) 50 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 25 nM catalytic subunit of cAMP-dependent protein kinase, and 10 μM γ-^{32}P-ATP (10–30 Ci/mmol). The samples were incubated at 30°C for 30 min, and the phosphorylation reaction terminated by addition of SDS-stop solution. The phosphoproteins were then separated by one-dimensional or two-dimensional gel electrophoresis and visualized by autoradiography of the dried gels. ^{32}P incorporation into specific protein bands was quantitated as described above. These incubation conditions were found to give maximal incorporation of ^{32}P into PCPP-260, as well as into synapsin Ia and Ib and Proteins IIIa and IIIb, nervous tissue-specific phosphoproteins which are known to be extracted with high efficiency by the acid-extraction technique (Huang et al., 1982; Ueda and Greengard, 1977). The incorporation of ^{32}P into these proteins, therefore, gives a quantitative measure of the proteins present in the extracts. However, the extent of phosphorylation of PCPP-260 in these extracts is not known, and the true amount of PCPP-260 in the tissues can therefore as yet not be determined with this technique. Nevertheless, the method can be used to compare PCPP-260 within individual experiments.

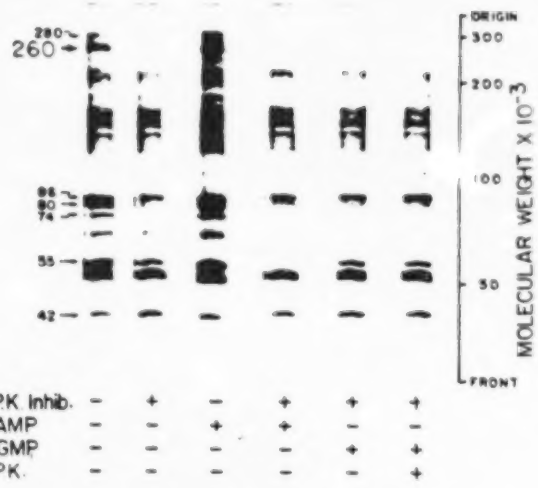

Figure 1. Autoradiogram showing phosphorylation of PCPP-260 by cAMP-dependent protein kinase. Aliquots containing equal amounts of total particular protein, prepared from rat cerebellum, were incubated with Mg γ-^{32}P-ATP in the absence or presence of the heat-stable protein inhibitor of cAMP-dependent protein kinase (*P.K. Inhib.*, 18 μg/ml), 8-bromo-cAMP (*8-Br-cAMP*, 2 μM), 8-bromo-cGMP (*8-Br-cGMP*, 2 μM), or the purified cGMP-dependent protein kinase (*cGMP-P.K.*, 10 nM), as indicated. After incubation at 30°C for 60 sec, the reactions were terminated by addition of SDS-containing stop solution and boiling, and the phosphoproteins were separated by SDS-PAGE on an 8% polyacrylamide gel and visualized by autoradiography. The *arrows* indicate the following proteins: 280, MAP-2; 260, cerebellum-specific 260,000 Da phosphoprotein (PCPP-260); 86, synapsin Ia; 80, synapsin Ib; 74, Protein IIIa; 55, Protein IIIb; 42, α-subunit of pyruvate dehydrogenase.

Results

cAMP-dependent phosphorylation of PCPP-260

In our original study reporting the existence of the 260 kDa phosphoprotein (PCPP-260) in rat cerebellum, we observed

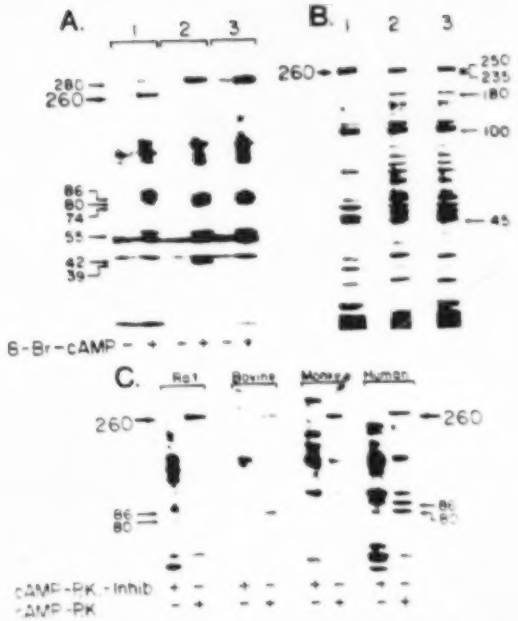

Figure 2. Distribution of PCPP-260 in mammalian brain. Total particulate fractions were prepared from rat cerebellum (*1*), neostriatum (*2*), and cerebral cortex (*3*), as described in the text. *A*, Aliquots containing equal amounts of protein were phosphorylated in the absence or presence of 8-bromo-cAMP (8-Br-cAMP, 2 μM), as described in the legend to Figure 1, and the phosphoproteins were separated by SDS-PAGE on a linear 6–12% gradient polyacrylamide gel and visualized by autoradiography. The *arrows* indicate as follows: 39, striatum-specific 39,000 Da phosphoprotein; others as in Figure 1. *B*, The proteins were separated on an 8% polyacrylamide gel for visualization by Coomassie blue. Numbers indicate molecular weight of major protein bands. *C*, Total particulate fractions from the cerebella of various mammalian species were phosphorylated as described above, with the addition of 0.2% Triton X-100 to the medium, in the absence or presence of the protein kinase inhibitor (18 μg/ml) or the catalytic subunit of cAMP-dependent protein kinase (20 nM), as indicated. The reactions were terminated and the phosphoproteins were separated and visualized as described above. Only the high-molecular-weight regions of the gels are shown. Phosphoproteins are indicated as above.

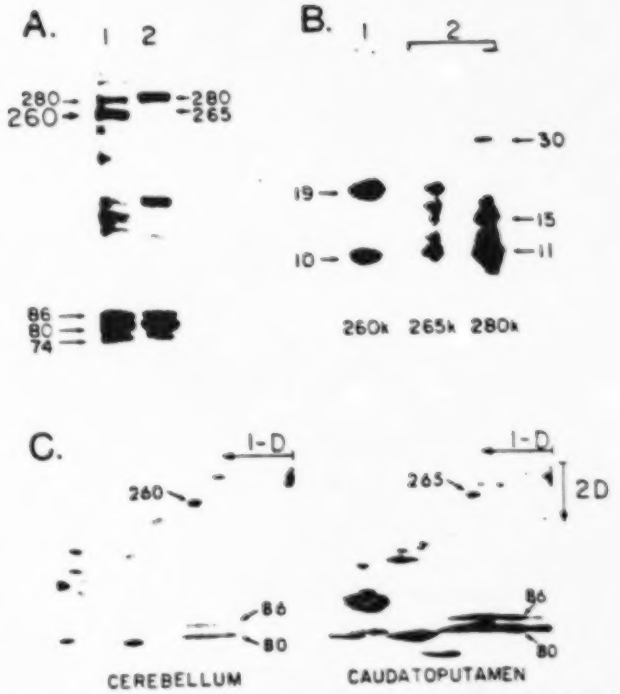

Figure 3. Demonstration of similarities between PCPP-260 and a 265 kDa forebrain protein. *A*, Total particulate preparations from neocortex (*1*) and cerebellum (*2*) were phosphorylated in the presence of 8-bromo-cAMP, as described in the legend to Figure 1, and the phosphoproteins were separated by SDS-PAGE on 6% acrylamide gels and visualized by autoradiography. A distinct difference in mobility between PCPP-260 (260) and the 265 kDa protein (265) can be observed, whereas both MAP-2 (280) and synapsin Ia and Ib (86 and 80) display identical mobilities in the two preparations. *B*, Peptide maps of the high-molecular-weight phosphoproteins shown in *A* demonstrating relationship between PCPP-260 and the 265 kDa protein. The indicated *bands* (260k, 265k, 280k) were cut out of the gel with the autoradiogram as guide and subjected to incomplete proteolysis with 1 μg of *S. aureus* V8 protease; the phosphopeptides were separated on 15% acrylamide gels and visualized as described in the text. *Numbers* indicate apparent molecular weight of the major phosphopeptide fragments generated from PCPP-260, the 265 kDa protein, or MAP-2 (280k). *C*, Two-dimensional electrophoresis of brain phosphoproteins, demonstrating similar mobilities of PCPP-260 and the 265 kDa protein. Total homogenates from rat cerebellum and caudatoputamen were phosphorylated in the presence of 8-bromo-cAMP, as described in the legend to Figure 1. Aliquots containing 250 μg protein from the caudatoputamen or 50 μg protein from the cerebellum were separated by two-dimensional electrophoresis, and the phosphoproteins were visualized by autoradiography. *1-D*, first dimension performed on urea-SDS-Triton CF-10 gels; *2D*, second dimension performed on 7–15% gradient SDS-PAGE. *Arrows* indicate as follows: 260, PCPP-260; 265, 265 kDa forebrain protein; 86 and 80, synapsin Ia and Ib. Only the parts of the gels containing high-molecular-weight proteins are shown in *A* and *C*.

phosphorylation of this protein in the absence and, to a greater extent, in the presence of added cAMP (Walaas et al., 1983a). The protein kinase responsible for phosphorylation in the absence of added cyclic nucleotide was not identified. In the present study, total particulate preparations from rat cerebelllum were used to characterize the protein kinase that catalyzes the phosphorylation of PCPP-260. Incubation of these preparations with γ-^{32}P-ATP and Mg^{2+} in the presence of 8-bromo-cAMP (Fig. 1) or the purified catalytic subunit of cAMP-dependent protein kinase (not shown) stimulated ^{32}P incorporation into PCPP-260 greater than that observed under basal conditions, whereas incubation with the purified heat-stable protein inhibitor of cAMP-dependent protein kinase led to an almost complete inhibition of ^{32}P incorporation into PCPP-260 (Fig. 1). Furthermore, the phosphorylation of microtubule-associated protein 2 (MAP-2) (280 kDa), as well as the synaptic vesicle-associated proteins, synapsin Ia and Ib (86 kDa and 80 kDa) and Proteins IIIa and IIIb (74 kDa and 55 kDa), all of which are well-known substrates for cAMP-dependent protein kinase (Browning et al., 1982; Huang et al., 1982; Ueda and Greengard, 1977; Walaas et al., 1983a), was regulated by these additions in a similar way (Fig. 1). These results indicate that PCPP-260 can be phosphorylated in particulate preparations by both intrinsic and exogenous cAMP-dependent protein kinase. The effect of the protein kinase inhibitor also suggests that the particulate preparation from rat cerebellum contains a partially activated endogenous cAMP-dependent protein kinase. The high postmortem levels of cyclic nucleotides known to occur in the rodent cerebellum may have been responsible for this activation (Corda et al., 1980; Ferrendelli et al., 1973).

cGMP-dependent protein kinase, calcium/calmodulin-dependent protein kinase, and calcium/phospholipid-dependent protein kinase are also present in the cerebellum (Bandle and Guidotti, 1978; Greengard and Kuo, 1970; Lohmann et al.,

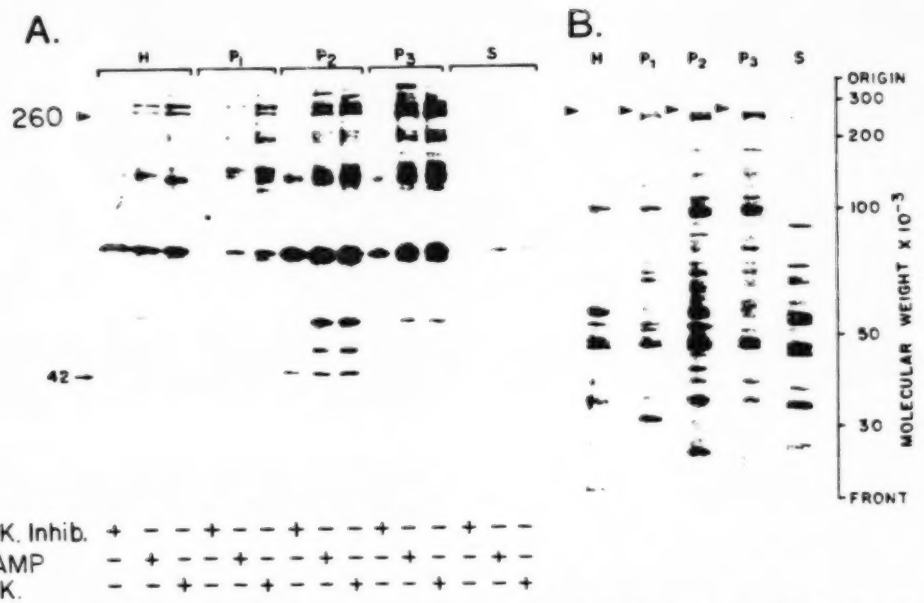

Figure 4. Distribution of PCPP-260 in various subcellular fractions from rat cerebellum. Cerebellar homogenates (*H*), and nuclear (P_1), crude synaptosomal (P_2), microsomal (P_3) and soluble fractions (*S*) were prepared and treated with Triton X-100, as described in the text. Aliquots containing equal amounts of protein were phosphorylated (as described in the legend to Fig. 1) in the presence of the protein kinase inhibitor (*P.K. Inhib.*, 18 μg/ml), 8-bromo-cAMP (*8-Br-cAMP*, 2 μM), or the catalytic subunit of cAMP-dependent protein kinase (*cAMP-P.K.*, 20 nM) as indicated. The phosphoproteins were then separated by SDS-PAGE on 8% acrylamide gels and visualized by autoradiography. *A*, Autoradiogram of phosphoproteins. *B*, Coomassie blue-stained protein bands. *Arrowheads*, PCPP-260, which is seen to be restricted to particulate fractions. *42*, α-Subunit of pyruvate dehydrogenase.

1981; Walaas et al., 1983a, b). Therefore, we investigated whether PCPP-260 could be phosphorylated by any of these protein kinases. Addition of 8-bromo-cGMP to cerebellar homogenates (not shown) and addition of 8-bromo-cGMP with or without exogenous cGMP-dependent protein kinase to particulate preparations (Fig. 1) did not lead to phosphorylation of the protein. Furthermore, activation of endogenous calcium/calmodulin-dependent or calcium/phospholipid-dependent protein kinase in cerebellar homogenates did not reproducibly stimulate the phosphorylation of any 260 kDa protein (not shown). These studies, then, suggest that PCPP-260 may be a specific substrate for cAMP-dependent protein kinase.

Distribution of PCPP-260

Since PCPP-260 is a substrate for cAMP-dependent protein kinase, phosphorylation of total particulate preparations from various parts of the rat brain in the absence or presence of 8-bromo-cAMP was used to study the distribution of the protein. Figure 2 shows that PCPP-260 is highly enriched in the cerebellum. Phosphorylation of samples from striatum, cerebral cortex (Fig. 2), or several other brain regions (Walaas et al., 1983a) failed to show the protein in other parts of the brain. A similar distribution of PCPP-260 was observed when samples were phosphorylated in the presence of added catalytic subunit of cAMP-dependent protein kinase (not shown). PCPP-260 was also seen when total particulate preparations from bovine, monkey, human (Fig. 2), or mouse cerebella were analyzed, but it was not detected when several non-nervous tissues were examined with this technique (not shown).

Comparison of the autoradiogram with the Coomassie blue-stained gels showed that PCPP-260 comigrated with a major cerebellum-enriched, protein-staining band on both one-dimensional (Fig. 2) and two-dimensional electrophoresis (not shown); evidence presented throughout this paper suggests that these two bands represent the same entity. Both the stained protein band and the phosphoprotein migrated on SDS-PAGE close to a 235–250 kDa doublet (Fig. 2), which probably represents the recently purified and characterized spectrin-like protein that has been designated fodrin (Levine and Willard, 1981) or calspectin (Kakiuchi et al., 1982).

Our experiments also showed the presence of other, more widely distributed particulate brain phosphoproteins, the distributions of which can be contrasted to that of PCPP-260. These include MAP-2, synapsin Ia and Ib, Proteins IIIa and IIIb, and the mitochondrial α-subunit of pyruvate dehydrogenase (42 kDa)—all of which are present throughout the CNS—and a distinct 39 kDa phosphoprotein enriched only in the neostriatum (Walaas et al., 1983a) (Fig. 2).

Close inspection of the autoradiograms also revealed a minor particulate phosphoprotein, which had an apparent M_r close to that of PCPP-260. This protein was seen in many forebrain regions, for example, the neocortex, caudatoputamen (Fig. 2), hippocampus, nucleus accumbens, olfactory tubercle, globus pallidus, and amygdala, but not in brain stem regions such as diencephalon, mesencephalon, pons/medulla, or the spinal cord (not shown). Samples from the neocortex, caudatoputamen, and cerebellum were used to study the relationship of this protein to PCPP-260 in more detail. Using gels with low acrylamide concentration, we found that this phosphoprotein consistently showed a lower electrophoretic mobility than PCPP-260 upon SDS-PAGE and that it had an apparent M_r of 265,000 (Fig. 3). However, peptide mapping of PCPP-260 and the 265 kDa phosphoprotein, using incomplete digestion with *S. aureus* V8 protease followed by SDS-PAGE, showed that the two proteins generated very similar phosphopeptides, with major phosphopeptide fragments of 10 kDa and 19 kDa being found in both samples. MAP-2, in contrast, gave major phosphopeptides of 30, 15, and 11 kDa (Fig. 3). Several minor phosphoproteins seen in the digest from the 265 kDa protein suggested that this protein might contain other components. However, separation

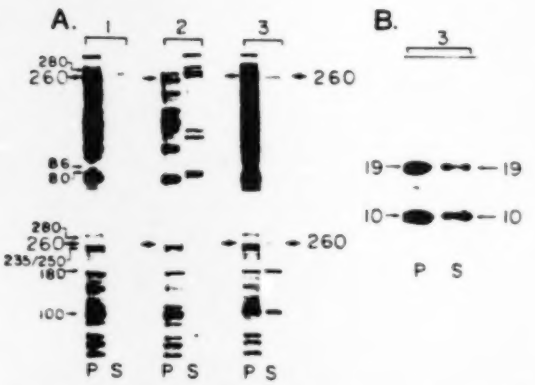

Figure 5. Extraction of PCPP-260 from microsomal fraction of rat cerebellum. *A*, Aliquots of the microsomal fraction (P_3) were resuspended in standard buffer containing no additions (*1*), 0.5 M NaCl (*2*), or Triton X-100 (0.5%, vol/vol) (*3*) and incubated for 60 min at 4°C. Solubilized proteins were removed by centrifugation and dialyzed overnight, while the pellets were resuspended in the original volume of standard buffer. Aliquots from supernatants and pellets representing equal amounts of starting material were then phosphorylated as described in the legend to Figure 1 in the presence of catalytic subunit of cAMP-dependent protein kinase (cAMP-P.K., 25 nM), and the phosphoproteins were separated by SDS-PAGE on 8% acrylamide gels and visualized by autoradiography. *Upper lane*, Autoradiogram of phosphoproteins. *Lower lane*, Coomassie blue-stained protein bands. Only high-molecular-weight proteins are shown. *B*, Autoradiogram of phosphopeptide fragments generated by limited proteolysis from particulate or solubilized PCPP-260 in the detergent-treated sample (*3*). Gel pieces containing PCPP-260 were cut out from the gel shown in *A* and subjected to limited proteolysis with 1 μg *S. aureus* V8 protease, as described in the legend to Figure 3. *P*, Nonextracted proteins; *S*, solubilized proteins. *Numbers* indicate apparent molecular weights of proteins in kilodaltons.

of proteins by two-dimensional gel electrophoresis showed that the 260 kDa and 265 kDa proteins had identical mobilities in the first-dimension electrophoresis (Fig. 3). In addition, two-dimensional tryptic peptide mapping of the two phosphoproteins cut out from these gels revealed identical phosphopeptides (not shown). Similarly, incomplete digestion of the 260 and 265 kDa phosphoproteins after acid/detergent extraction showed identical peptide maps (Fig. 6*C*; see below). Thus, the 265 kDa forebrain phosphoprotein is closely related to PCPP-260.

Subcellular localization of PCPP-260

Phosphorylation by endogenous or exogenous cAMP-dependent protein kinase was used to analyze various subcellular fractions from rat cerebellum for the presence of PCPP-260. Figure 4 shows that PCPP-260, visualized by autoradiography and by Coomassie blue staining, is restricted to the particulate fractions. Indeed, the protein appears to be enriched in the microsomal fraction (P_3), which contains predominantly plasma membrane fragments and microsomes (Whittaker and Barker, 1972). Similar results were obtained when acid-detergent extracts from these fractions were analyzed (not shown). This distribution can be contrasted with that of the 42 kDa phosphorylated α-subunit of the mitochondrial enzyme, pyruvate dehydrogenase (Walaas et al., 1983a). The latter phosphoprotein is enriched in the crude synaptosomal fraction (P_2), which contains the mitochondria (Whittaker and Barker, 1972), but it is absent from the microsomal fraction (Fig. 4).

These results indicate that PCPP-260 represents a membrane-bound protein entity. Therefore, we investigated whether the protein behaved as a peripheral or an integral membrane protein. Treatment of microsomal or total particulate preparations with a nonionic detergent, both at neutral and at acid pH, led to a partial solubilization of PCPP-260, as visualized by both autoradiography and protein staining (Fig. 5). In contrast, treatment with high salt concentrations (Fig. 5) or with acid in the absence of detergent did not solubilize the protein. Peptide maps confirmed that the detergent-solubilized phosphoprotein was identical to the particulate phosphoprotein (Fig. 5). Therefore, PCPP-260 behaved like an integral membrane protein. Similar results were obtained with the 265 kDa protein in forebrain samples (not shown). However, certain other proteins present in the particulate fractions, such as MAP-2, were most efficiently extracted by high concentrations of salt, thus behaving like peripheral membrane proteins (Fig. 5).

Cellular localization of PCPP-260 within the cerebellum

Cerebella from normal mice and from strains of mutant mice deficient either in Purkinje cells or granule cells were analyzed to determine the cellular localization of PCPP-260. Total particulate preparations or acid-detergent extracts were used to compare the phosphoproteins in the cerebellum, caudatoputamen, and neocortex of these mouse mutants, using both one- and two-dimensional gel electrophoresis and peptide mapping to identify the proteins. Figure 6 illustrates that PCPP-260 was present in the cerebella of control mice, but was essentially absent from the cerebella of homozygous PCD mice, which were almost devoid of Purkinje cells (Mullen et al., 1976). Similarly, PCPP-260 was greatly decreased in the cerebella of homozygous Nervous mice, which have lost most, but not all, Purkinje cells (Sidman and Green, 1970). In contrast, there was an increased concentration of PCPP-260 in the cerebella of the homozygous

Table 1. Concentrations of PCPP-260, synapsin I, Protein IIIa, and Protein IIIb in extracts from cerebella of neurologically mutant mice and their littermate controls

Phospho-protein	Genotype					
	Purkinje cell degeneration		Nervous		Weaver	
	+/? (n = 10)	−/− (n = 8)	+/? (n = 3)	−/− (n = 3)	+/? (n = 6)	−/− (n = 6)
PCPP-260	2.12 ± 0.36	0.08 ± 0.04*	3.35 ± 0.78	0.64 ± 0.12*	3.46 ± 0.97	6.30 ± 1.08**
Synapsin I	19.08 ± 1.97	12.60 ± 0.95**	18.55 ± 3.10	12.80 ± 2.35**	20.29 ± 1.43	10.15 ± 4.32*
Protein IIIa	2.08 ± 0.45	1.75 ± 0.34	1.43 ± 0.26	n.d.	2.09 ± 0.38	1.54 ± 0.21***
Protein IIIb	4.85 ± 0.54	3.88 ± 0.58***	2.71 ± 0.31	n.d.	3.84 ± 0.59	2.38 ± 0.62***

Cerebella were weighed, and acid-detergent extracts containing identical amounts of protein were prepared and phosphorylated with γ-^{32}P-ATP and the catalytic subunit of cAMP-dependent protein kinase. Phosphoproteins were separated by SDS-PAGE, visualized by autoradiography, cut out from the gels, and quantitated by liquid scintillation counting as described in the text. Results represent pmol ^{32}P incorporated into the specific proteins per mg protein in the neutralized extracts, and are presented as mean ± SD. Mean cerebellar weights in the different groups: PCD − +/?, 57 mg; −/− −46 mg; Nervous − +/?, 53 mg; −/−, 48 mg; Weaver − +/?, 55 mg; −/−, 19 mg.

Significance of difference between control (+/?) and homozygous (−/−) mice tested with Wilcoxon's two-sample test (Wilcoxon, 1945): *, $p < 0.001$; **, $p < 0.01$; ***, $p < 0.05$.

n.d. = not determined.

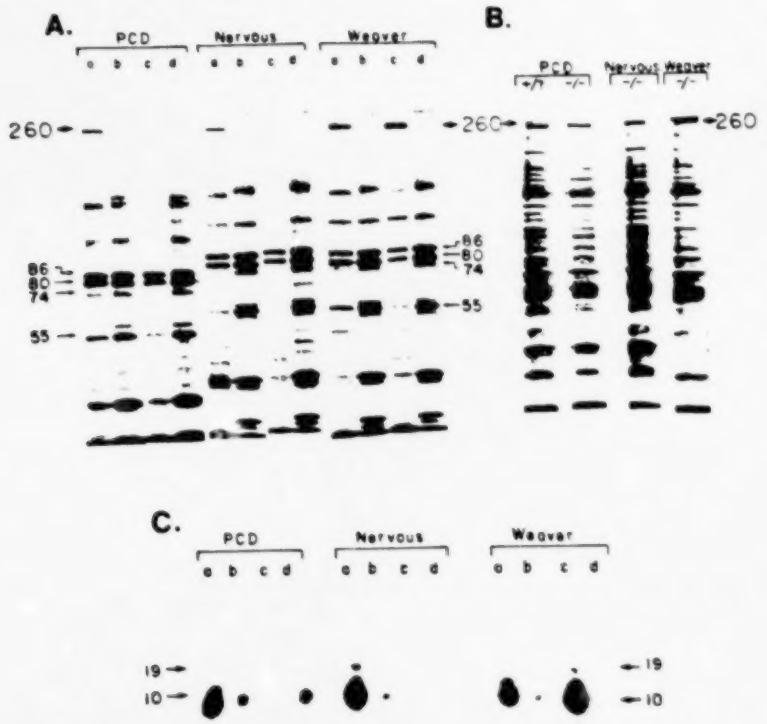

Figure 6. Distribution of PCPP-260 in cerebellum and neostriatum of normal and mutant mice. *A*, Acid-detergent extracts of total cerebellar (*a*, *c*) and neostriatal (*b*, *d*) homogenates were prepared from Purkinje cell-deficient (PCD), Nervous, and Weaver mice and their respective control littermates, and phosphorylated in the presence of catalytic subunit of cAMP-dependent protein kinase, as described in the text. After maximal ^{32}P incorporation had been achieved, the phosphoproteins were separated by 6–10% gradient SDS-PAGE and visualized by autoradiography. *a*, Sample from control cerebellum; *b*, sample from control neostriatum; *c*, sample from mutant cerebellum; *d*, sample from mutant neostriatum. *B*, equal amounts of total particulate proteins from cerebella of *PCD* control littermate (+/?), *PCD* mutant (−/−), *Nervous* mutant (−/−), and *Weaver* mutant (−/−) were separated by SDS-PAGE on 8% acrylamide gels, and the proteins visualized by Coomassie blue staining. PCPP-260 can be seen to be absent or decreased in the PCD and Nervous mutants, but increased in the Weaver mutant. *C*, Gel pieces containing phosphoproteins of 260–265 kDa were cut from the gels shown in *A*, and processed for limited proteolysis with 10 μg *S. aureus* V8 protease, as described in the legend to Figure 3. This amount of protease generated predominantly the 10 kDa fragment, which can be seen to be absent from the PCD mutant cerebellum, but present in all other samples.

Weaver mice, which have lost the large population of granule cells and parallel fibers, but have retained the Purkinje cells (Table 1; Rakic and Sidman, 1973).

Calculations of the total amounts of PCPP-260 indicated that the cerebella of homozygous PCD mice contained approximately 3.1%, the cerebella of homozygous Nervous mice 17%, and the cerebella of homozygous Weaver mice 63% of the total amounts of PCPP-260 found in their respective control littermates (mean values, calculated from Table 1).

In comparison, the concentrations of synapsin I, Protein IIIa, and Protein IIIb, phosphoproteins present in virtually all nerve terminals and therefore useful as general neuronal markers (DeCamilli et al., 1983), were decreased by only 25–35% in the PCD and Nervous homozygous mutants. The Weaver mutants showed a more pronounced decrease (Table 1), which represented a loss of approximately 80% of the total amount of these proteins, in agreement with previous reports (e.g., Dolphin and Greengard, 1981). No significant changes were observed in the amounts of synapsin I, Protein IIIa, or Protein IIIb in the caudatoputamen (Fig. 6) or neocortex (not shown) in any of these mutants.

The 265 kDa phosphoprotein was barely detectable upon SDS-PAGE of phosphorylated acid-detergent extracts from forebrain regions (Fig. 6). However, peptide mapping and two-dimensional gel electrophoresis allowed us to analyze this protein in the caudatoputamen and neocortex of the mutant mice. These studies showed that neither the caudatoputamen nor the neocortex had any significant change in the amount or concentration of 265 kDa protein in any of the mutants (Fig. 6; data not shown).

These results, therefore, indicate that, within the cerebellum, PCPP-260 is highly enriched in and may be specific to Purkinje cells. In contrast, many forebrain regions contain small amounts of a closely related 265 kDa phosphoprotein, which appears to be located in cells that are insensitive to the PCD, Nervous, and Weaver mutations.

Discussion

The 260 kDa phosphoprotein (PCPP-260) analyzed in this paper appears to be specifically concentrated in cerebellar Purkinje cells. The protein has been found in high concentrations in all mammalian cerebella tested so far, and studies on mutant mice indicate a unique enrichment in Purkinje cells. Furthermore, it can be phosphorylated in broken cell preparations by cAMP-dependent protein kinase, but apparently not by cGMP-, calcium/calmodulin-, or calcium/phospholipid-dependent protein kinases. This set of properties, together with data indicating that it is an integral membrane protein, suggest that the protein is distinct from certain other high-molecular-weight brain phosphoproteins that have been characterized—for example, the more

widely distributed protein MAP-2, the microtubule-associated protein, which is a substrate for cAMP-dependent, calcium/calmodulin-dependent, and calcium/phospholipid-dependent protein kinases (Walaas et al., 1983b). However, PCPP-260 is apparently closely related to a minor 265 kDa phosphoprotein present throughout the telencephalon, since the two proteins share subcellular localization, extractability, protein kinase specificity, electrophoretic mobility upon mixed detergent-urea gel electrophoresis, and one- and two-dimensional phosphopeptide maps, but exhibit slight differences in mobility upon SDS-PAGE. An understanding of the exact relationship between these two proteins requires further study.

Protein staining of the cerebellar proteins after one- or two-dimensional electrophoresis suggests that PCPP-260 may constitute a major component of the Purkinje cells, since the phosphoprotein has the same molecular mass and electrophoretic mobility as a distinct Coomassie blue-stained band. This stained protein band is probably identical to the phosphoprotein, since they share brain distributions, are absent from cerebella deficient in Purkinje cells, are enriched in cerebella deficient in granule cells, and have similar subcellular localization and extraction properties.

Previous studies have indicated the existence of a cerebellum-specific glycoprotein with an apparent M_r of 400,000, which was designated P_{400} (Mallet et al., 1976). This protein appears to have a localization similar to that of PCPP-260, since it was found to be restricted to particulate fractions from Purkinje cells (Mallet et al., 1976). Subsequent immunocytochemical studies suggested that P_{400} was present on Purkinje cell dendrites and cell bodies (Mikoshiba et al., 1979). Other investigators have reported the presence of a membrane glycoprotein with apparent M_r of 240,000, which was stated to be restricted to the cerebellum, enriched in synaptic junctions, and partly solubilized by nonionic detergents in the absence of calcium (Groswald and Kelly, 1984; Groswald et al., 1983). A similar glycoprotein of M_r 250,000 was also found in low concentrations in the forebrain (Aono et al., 1983). The similarities in regional, cellular, and subcellular distribution between the glycoproteins (P_{400} and the 240–250 kDa proteins) and PCPP-260 suggest that the proteins may be identical, with differences in the molecular weight estimates being the result of the different SDS-PAGE systems used. However, P_{400} has been stated to be insoluble in high concentrations of detergents such as Triton X-100 and deoxycholate (Mikoshiba et al., 1979), in contrast to PCPP-260, which is partly soluble in nonionic detergents. Therefore, the exact relationship between PCPP-260 and the previously described glycoproteins remains to be established.

The well-defined and relatively simple architecture of the mammalian cerebellum (Eccles et al., 1967; Llinas, 1975; Palay and Chan-Palay, 1974) has made this region of the brain particularly attractive for neurochemical and physiological studies. Such studies have revealed that the cAMP-regulated and cGMP-regulated protein phosphorylation systems in the cerebellum are differently distributed. cGMP-dependent protein kinase and its substrate (G-substrate) are restricted to the cytosol of Purkinje cells, suggesting a unique functional role for cGMP in this neuron, whereas the cAMP-regulated system is more widely distributed in many cell types, indicating a more widespread role for this second messenger in the cerebellum (Bandle and Guidotti, 1978; Detre et al., 1984; Dolphin et al., 1983; Lohmann et al., 1981; Schlichter et al., 1980). The results described in this paper suggest that the cAMP-regulated phosphorylation of PCPP-260, a Purkinje cell-enriched phosphoprotein, may also play a unique and important functional role in the cerebellar Purkinje cell.

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